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Thermophilin 110: A Bacteriocin of *Streptococcus thermophilus* ST110

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Abstract. A screen of thermophilic lactic acid bacteria identified *Streptococcus thermophilus* strain ST110 as the putative producer of a bacteriocin with high level of activity against pediococci. Thermophilin 110 was isolated from culture supernatant after 16 h of growth and partially purified by a chloroform extraction procedure. The bacteriocin inhibited the growth of several lactic acid bacteria and in the case of *Pediococcus acidilactici*, it induced cell lysis with the concomitant release of OD_{260} -absorbing material and intracellular enzymes. SDS-PAGE analysis revealed two components with estimated sizes between 4.0 kDa and 4.5 kDa, respectively, with possible involvement in bacteriocin activity as indicated by agar overlay assays with *P. acidilactici* as the target organism. Thermophilin 110 was inactivated by several proteolytic enzymes and also by α-amylase, which indicated the putative requirement of a glycosidic component for activity. The bacteriocin produced by *S. thermophilus* may be especially useful in the food processing industries to control spoilage caused by pediococci.

Bacteriocins are a group of small proteins that exhibit bactericidal activity usually towards strains closely related to the producer culture. They are a varied group of ribosomally synthesized proteins divided into three different classes [18]. Briefly, Class I bacteriocins are the lantibiotics, which are post-translationally modified to incorporate unusual amino acids. Lantibiotics include both one-peptide and two-peptide bacteriocins. Class II bacteriocins are small, cationic, heat-stable proteins whereas Class III includes large heat-labile antimicrobial proteins. Most bacteriocins fall into class II, which has narrow-range antimicrobial activity and is subdivided into four groups. Class IIa bacteriocins, which are known for strong antilisterial activity, include the well-characterized bacteriocin pediocin PA-1 [23]. Class IIb bacteriocins include the two-component peptides. Most class IIa and IIb bacteriocins are synthesized as prepeptides with a double-glycine type of leader sequence that is enzymatically removed during secretion by a dedicated ABC transporter [15]. In the case of class IIc bacteriocins, the leader peptide is removed by a secretion (*sec*-)-dependent pathway. The last group, class IId, consists of bacteriocins that do not belong to the other subgroups of Class II bacteriocins.

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Because bacteriocins are naturally produced, there is much interest in using them to control the growth of pathogens or spoilage organisms in food products and food fermentations [35]. Bacteriocins produced by food-related lactic acid bacteria (LAB) are of particular interest because of established safe status in food fermentations [9]; also, nisin, a lantibiotic-class bacteriocin of *Lactococcus lactis* subsp. *lactis*, has been approved for food use in over 40 countries including the United States. Although the production of bacteriocins in *Streptococcus thermophilus* has been reported by several laboratories [2, 16, 20, 25, 37, 38], as a group, food-grade thermophilic lactic streptococci, which are essential starter cultures in yogurt and some cheese fermentations, remain a largely untapped source of bacteriocins. In this report, we describe the characterization of an apparently two-component bacteriocin produced by *Streptococcus thermophilus* ST110.

Materials and Methods

Bacteria and growth conditions. The bacteriocin-producing *S. thermophilus* ST110, originally isolated from raw milk, was

Table 1. Antimicrobial spectrum of thermophilin 110 from *Streptococcus thermophilus* ST110

Test organism	Number of strains tested	Number of sensitive strains	Range of inhibition zones (mm)
Streptococcus thermophilus	34	27	8–14
Lactococcus lactis ssp. *Lactis*	16	16	8–11
Lactobacillus delbrueckii ssp. *bulgaricus*	8	2	12
Pediococcus acidilactici	8	8	9–18
P. pentosaceus	4	4	8–11
P. dextrinicus	1	1	15
P. inopinatus	1	1	10
P. parvulus	1	1	10
P. damnosus	3	1	14
Enterococcus faecalis	1	1	8
Listeria monocytogenes	5	5	7–9
Staphylococcus epidermidis	7	0	
S. aureus	2	0	

Diameter of well: 5 mm. Sample volume: 50 μL per well.

maintained in tryptone-yeast extract-lactose (TYL) broth, pH 6.5 at 37°C [33], and its identity was verified by 16S rRNA gene sequence analysis (MIDI Labs, Inc., Newark, DE). Other strains of *Streptococcus thermophilus*, *Lactococcus lactis* subsp. *lactis*, and *Lactobacillus delbrueckii* subsp. *bulgaricus* listed in Table 1 were from an in-house collection [38]. *Pediococcus acidilactici* strain F was a gift from B. Ray (University of Wyoming), whereas strains of all other cultures were obtained from the culture collection of the National Center for Agricultural Utilization Research (Peoria, IL) and the American Type Culture Collection (Manassas, VA). Pediococci and lactobacilli were grown in deMan, Rogosa, and Sharpe medium (MRS, Becton Dickinson, Sparks, MD) at 30°C and 37°C, respectively. Staphylococci and enterococci were grown in tryptic soy broth (TSB, Becton Dickinson) at 37°C, whereas *Listeria monocytogenes* was maintained in Brain Heart Infusion (BHI, Becton Dickinson) medium at 30°C.

Bacteriocin assays. Bacteriocin production by *S. thermophilus* ST110 was demonstrated by an agarose/agar overlay assay with several target organisms [34]. In these tests, spread plates of ST110 were prepared using mixed agarose/agar films, followed by incubation at 37°C until colonies were 0.8–1 mm in diameter. The films were layered on top of agar plates seeded with the target microbes, and zones of inhibition surrounding ST110 colonies were checked after 24 h. Bacteriocin produced by *S. thermophilus* ST110 was measured by a modified agar diffusion method [36]. Cell-free samples of a culture growing in TYL medium for 18 h at 37°C were prepared by serial twofold dilution with sterile distilled water, and 50 μL of each sample was deposited in wells (5-mm diameter) cut into MRS agar medium seeded with *P. acidilactici* F. One thermophilin 110 activity unit (TAU) was defined as the highest dilution showing a zone of inhibition of the indicator organism. Total bacteriocin activity of samples was calculated as the reciprocal of the highest dilution and expressed as TAU mL^{-1}.

Chloroform precipitation of thermophilin 110. An overnight culture of *S. thermophilus* ST110 was used to inoculate 400 mL of TYL medium at 1% and grown at 37°C for 18 h. The cells were collected by centrifugation in a GSA rotor at 9000 rpm for 15 min at 4°C. The bacteriocin was extracted from the supernatant as described by Burianek and Yousef [8]. Briefly, 200 mL of chloroform was added to 400 mL supernatant, and the mixture was stirred vigorously at room temperature for 45 min. The mixture was transferred to centrifuge bottles and spun again for 20 min in a GSA rotor at 9,000 rpm at 4°C. Both aqueous and solvent phases were carefully decanted and discarded. The sediments deposited on the sides and bottom of the tubes or formed at the interface were dispersed in 15 mL sterile distilled water and centrifuged again as described above to separate the remaining solvent from the water and sediment. The remaining traces of solvent were driven off by a stream of air. After storage at 4°C overnight to allow for protein renaturation, the samples were centrifuged as described above, the aqueous layers were decanted and set aside, and sediments were re-extracted with 5 mL dH_2O. The cell-free supernatant before and after chloroform extraction, the aqueous layer resulting after chloroform extraction, and the precipitates resulting from chloroform treatment were all tested for bacteriocin activity against *P. acidilactici* F as the test organism.

Effect of enzymes and heat treatment. Samples of partially purified thermophilin 110 were tested for loss of activity following treatment with digestive enzymes from a commercial source (SIGMA). Stock solutions (1 mg/mL) of *Bacillus subtilis* protease, papain, proteinase K, chymotrypsin, trypsin, pronase, leucine aminopeptidase, carboxypeptidase, lysozyme, α-amylase, and aminoglucosidase were prepared in 20 mM sodium phosphate buffer (pH 6.0), whereas pepsin was prepared in distilled water. Double rows of wells were cut into MRS agar gels inoculated with *P. acidilactici* F in rectangular plates. Fifty microliters of each enzyme solution was pipetted into wells of the bottom rows, and plates were kept at 6°C for 4–6 h to allow for diffusion. Then 50 μL of the partially purified thermophilin 110 solution was placed into each well of the top rows and the plates were again held at 6°C for 4 h before transfer to an incubator set at 32°C. After incubation for 16 h, the plates were examined for changes in the appearance of inhibition zones.

To test for thermal stability, the partially purified thermophilin 110 was held in a water bath at 100°C and aliquots were taken at 5, 10, 15, 30, 45, 60, 90, and 120 min for agar diffusion assays.

Mode of inhibition. Cultures were tested for sensitivity to thermophilin 110 by inoculating 9.5 mL of medium with 50 μL of an overnight culture of the test organism and incubating to attain an absorbance of 0.5 at OD_{660}. At this point, 500 μL of partially purified thermophilin 110 was added to each tube and changes in absorbance were followed for up to 6 h. In addition, cells from a culture of *P. acidilactici* (OD_{660} = 0.5) were removed by centrifugation, and cells

resuspended in 9.5 mL phosphate buffer were mixed with 0.5 mL of thermophilin 110. Absorbance was monitored at OD_{660} and OD_{260} to follow changes in cell density and the release of intracellular material, respectively. Cell lysis was also monitored by checking cell-free samples (100 μL) for the presence of lactate dehydrogenase (LDH) activity, by following the decrease in absorbance at 340 nm resulting from the pyruvate-dependent oxidation of NADH [39] in a Beckman spectrophotometer.

Gel electrophoretic analysis of thermophilin 110. The proteins were visualized by polyacrylamide gel electrophoresis (PAGE) in the presence of SDS using the Invitrogen (Carlsbad, CA) Xcell SureLock Minicell system and Nu-PAGE Novex precast gels, according to the manufacturer's specifications. The samples were reduced with DTT and mixed with LDS sample buffer, then heated at 95°C for 5 min. NuPAGE antioxidant was added to the upper chamber to prevent reoxidation of the samples. The samples were loaded on the 12% Bis-Tris gel next to a Mark 12 ladder (Invitrogen, Carlsbad, CA), and run with MES-SDS buffer at neutral pH. The gels were run for 30 min at 200 V, stained with Coomassie blue R-250 for 30 min, and destained overnight.

Gel overlay assays were performed as described by Bhunia et al. [6]. After the fixation of proteins for 2 h followed by washing with water overnight, the gel was placed in a Petri dish and overlaid with MRS agar seeded with *P. acidilactici*. The plates were incubated overnight at 34°C and examined for inhibition zones.

Glycoprotein gels were run using the Invitrogen system described above with the sample and the CandyCane Glycoprotein molecular weight standard and then stained with the GelCode Glycoprotein Staining kit from Molecular Probes (Eugene, OR), according to the manufacturer's specifications. The protein bands were fixed in a 50% methanol–5% acetic acid solution, followed by washing in 5% acetic acid. The gel was oxidized with periodic acid (2.5 g) in 250 mL of 3% acetic acid and then washed again. The gel was stained with the Pro-Q Emerald 300 (acidic fuchsin sulfite in dimethylformamide) staining solution for 2 h in the dark. The gels were then washed again until the background was clear enough to allow visualization of stained bands with a UV light source at 300 nm.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The water-soluble portion of a sample obtained by chloroform extraction was further purified by size exclusion column chromatography on a column of G-25 Sephadex (2.5 × 40 cm) equilibrated with water. With water used as the mobile phase, fractions (1 mL) were collected at a flow rate of 20 mL per hour and tested for antimicrobial activity by the agar well diffusion assay. Active fractions were pooled and analyzed in a model 4700 Proteomics Analyzer Instrument (Applied Biosystems, Framingham, MA) in the positive reflectron mode with a 200 Hz Nd-YAG 355 laser. Conversion of time-of-flight (TOF) to mass (Da) for the monoisotopic ions, $[M + H]^+$ was based on calibration of the instrument with a peptide standard kit (Applied Biosystems) that included des-Arg^1-bradykinin (*m/z* 904), angiotensin I (*m/z* 1,296), Glu^1-fibrinopeptide B (*m/z* 1,570), ACTH (1–17, *m/z* 2,903), ACTH (18–39, *m/z* 2,465), and ACTH (7–38, *m/z* 3,657). Peptides were extracted using a C18 ZipTip, washed with water containing 0.1% trifluoroacetic acid (TFA), re-extracted with acetonitrile-water (1:1) 0.1% TFA, and mixed with a recrystallized α-cyano-4-hydroxycinnamic acid or sinapinic acid matrix solution (5 mg/mL, acetonitrile-water- (1:1) 0.1% TFA) to a final concentration between 100 fmol and 1 pmol/μL. Approximately 0.6–0.7 μL of the peptide-matrix solution was spotted on the MALDI target plate.

Compositional analyses. The overnight culture of *S. thermophilus* ST110 was analyzed for the presence of plasmid DNA by a protocol previously described [33]. The protein and carbohydrate content of the thermophilin 110 preparation prepared by chloroform precipitation was estimated by the method of Lowry et al. [19] and Dubois et al. [13], respectively.

Results

Antimicrobial spectrum. Inhibition of several target organisms by *S. thermophilus* ST110 was demonstrated by the agarose/agar overlay assay and is shown in Fig. 1. Data on the antimicrobial spectrum of crude bacteriocin present in cell-free culture filtrates of *S. thermophilus* ST110 are summarized in Table 1. Thermophilin 110 was inhibitory to most related strains within the species and also inhibited representative strains in the genera *Lactococcus*, *Lactobacillus*, *Enterococcus*, and *Pediococcus*, but had no activity against strains of *Staph. aureus* and *Staph. epidermidis*. Activity displayed against strains in the *Listeria* group was slight, and several inhibition zones became overgrown after 48-h incubation, indicating the development of resistance in target organisms.

Thermophilin 110 production. The kinetics of thermophilin 110 production was monitored during the growth of *S. thermophilus* ST110 in TYL broth at 37°C. Bacteriocin activity was first measurable after 4 h, reached a maximum after 6 h of incubation (640 TAU/mL), and remained stable for at least 2 additional hours (Fig. 2), while medium pH dropped from an initial value of 6.5 to 5.0. However, antimicrobial assays indicated an approximately 50% decline in thermophilin 110 activity in cell-free filtrates prepared after 24 h of growth. The presence of plasmid DNA in the producing culture could not be demonstrated.

Effect of enzymes and heat treatment. Tests on the effect of enzymes and heat treatments showed that thermophilin 110 was susceptible to digestion by most proteases and was also inactivated by α-amylase (Fig. 3). Thermophilin 110 remained stable when held at 100°C for 60 minutes but lost its activity against *P. acidilactici* F after 90 minutes or longer exposure (data not shown).

Purification of thermophilin 110. The results of the chloroform extraction-based purification procedure are summarized in Table 2. Overall, the solvent extraction method resulted in a 40% recovery of the initial total activity and a 522-fold increase in the specific activity of thermophilin 110.

SDS-PAGE analysis on the Nu-PAGE Novex gels indicated the presence of two components in thermophilin 110 obtained by chloroform precipitation. The

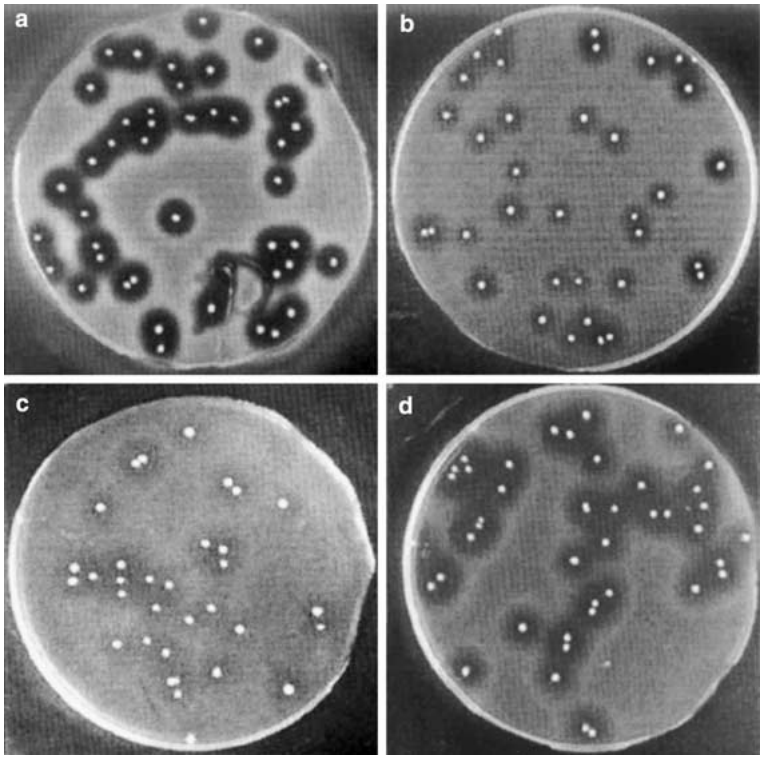

Fig. 1. Bacteriocin production by *S. thermophilus* ST110. Agar plates seeded with target organisms were overlaid with agarose/agar spread plates of a 36-h *S. thermophilus* ST110 culture. Zones of inhibition were checked after 24 h. Targets: *P. acidilactici* F (a), *P. dextrinicus* ATCC 700477 (b), *P. damnosus* ATCC 43013 (c), *S. thermophilus* ST113 (d).

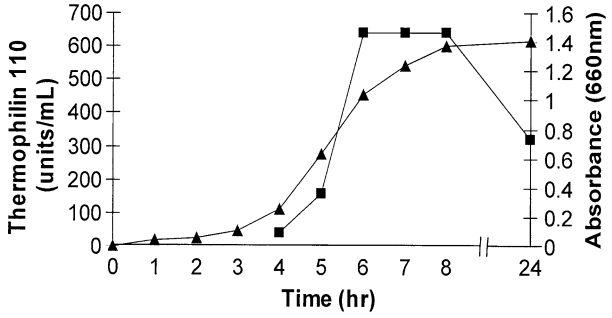

Fig. 2. Production of thermophilin 110 during growth of *Streptococcus thermophilus* ST110 in TYL medium at 37°C. (▲) Optical density at 660 nm; (■) activity of thermophilin 110 in TAU/mL.

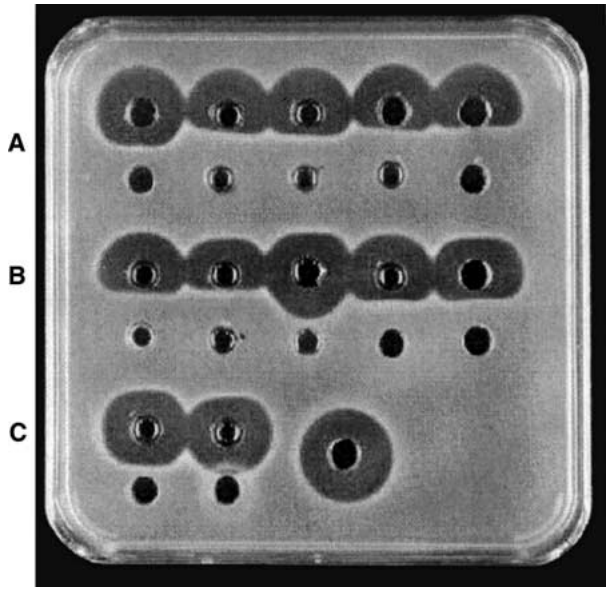

Fig. 3. Sensitivity of thermophilin 110 to enzyme treatments. (A) Upper row: thermophilin 110; lower row (*left to right*): pepsin, trypsin, papain, pronase, proteinase K. (B) Upper row: thermophilin 110; lower row (*left to right*): *Bacillus subtilis* protease, chymotrypsin, leucine aminopeptidase, carboxypeptidase, α-amylase. (C) Upper row: thermophilin 110; lower row (*left to right*): amyloglucosidase, lysozyme; last well was the control with undigested thermophilin 110. Target organism: *P. acidilactici* F in MRS agar (1.5%).

estimated size of each of the components was between 4 kDa and 5 kDa. The putative peptide bands also picked up the glycoprotein-specific stain, which confirmed the presence of a glycosidic moiety (Fig. 4). The overlay assay of the gel with *P. acidilactici* as the target organism gave a visible zone of inhibition and indicated the involvement of at least one or two bands in the display of antimicrobial activity by thermophilin 110 from *S. thermophilus* ST110 (Fig. 5).

MALDI-TOF-MS of thermophilin 110 further purified by Sephadex G-25 chromatography identified

Table 2. Purification of thermophilin 110

Purification step	Volume (mL)	Activity (AU/mL)	Total activity (AU)	Protein (mg/mL)	Specific activity	Recovery (%)	Purification (fold)
Culture supernatant	400	640	256,000	22	30	100	1
Pooled $CHCl_3$ extracts	20	5,120	102,400	0.32	15,657	40	522

Activity is calculated by the agar-well diffusion assay; sample volume: 50 μL per well. Total activity is determined by the multiplication of volume with activity. Protein concentration is determined by the Lowry method. Specific activity is activity units divided by the protein concentration (AU/mg). Recovery is the final total activity as a percentage of the initial total activity. Purification fold is the increase in the initial specific activity.

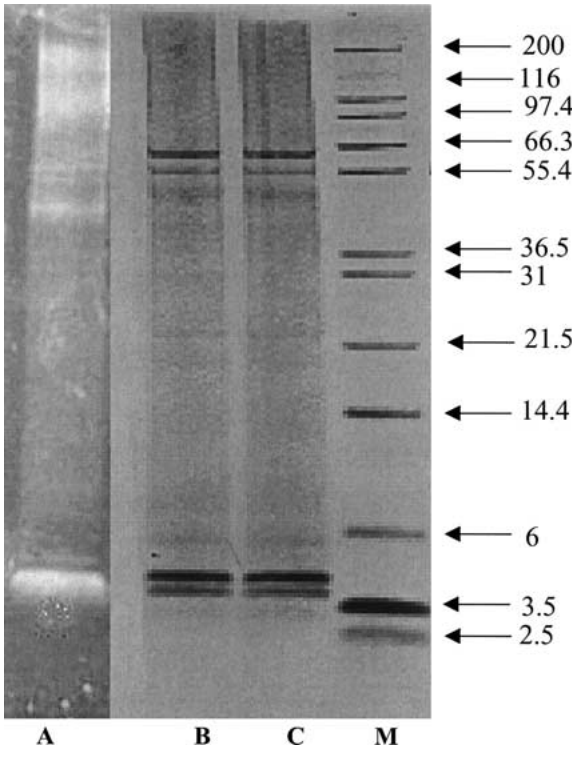

Fig. 4. SDS-PAGE gels of thermophilin 110 prepared by the chloroform extraction procedure. (A) Gel treated with glycoprotein-specific stain. (B) and (C) Coomassie blue-stained gel of two different lots of thermophilin 110 preparations. (M) Protein standards of known molecular weights (kDa).

three putative peptide components based on the presence of peaks with *m/z* values (*m/z* value is the mass/charge ratio, where *z* is usually 1) of 5,139, 5,285, and 5,776, respectively. Although the estimated molecular size of each peptide detected by MALDI-TOF-MS fell within the estimated size range of the biologically active peptides identifiable by SDS-PAGE analysis (ca. 5,000 Da) and overlay agar diffusion assay, a direct relationship among these peptides could not be established.

Mode of action. In studies designed to characterize its mode of antimicrobial activity, thermophilin 110 was observed to induce cell lysis in several pediococci. To explore the lysis phenomenon further, *P. acidilactici* F was grown to an absorbance of 0.5 at OD_{660}, with the cells centrifuged and resuspended in 20 mM potassium phosphate buffer (pH 6.0). Partially purified thermophilin 110 was added to the cell suspension. Absorbance values were checked at 660 nm and 260 nm over a 2 h period to follow changes in cell density and to detect the release of intracellular material (Fig. 6). As the cell density decreased, the OD_{260} readings increased indicating cell lysis. After about 2 h, the culture was completely lysed and the OD_{660} was close to zero while the OD_{260} increased to 0.8. Since cells apparently were lysing, experiments were carried out to measure the presence of the intracellular enzyme LDH in cell-free supernatant fluids. Samples were taken at various time points, the cells were removed, and the supernatant was stored at 4°C until they were assayed for the presence of LDH. As cell lysis progressed, the level of LDH in the supernatant increased, resulting in the oxidation of NADH and the decrease in OD_{340} values.

Carbohydrate content of thermophilin 110. On the basis of spectrophotometric assays, the total carbohydrate content (using a glucose standard) of partially purified thermophilin 110 was estimated as 16% by weight. This amount of carbohydrate corresponded to 4 to 5 mole equivalent of hexose (as glucose) per mole of thermophilin 110 (based on an estimated size of 4–5 kDa).

Discussion

Thermophilin 110, an apparently novel bacteriocin, was isolated from *Streptococcus thermophilus* ST110 with a spectrum of activity that included related strains of *S. thermophilus*, *P. acidilactici*, and other pediococci as well as lactococci. The bacteriocin was also active against selected strains of *L. delbrueckii* subsp. *bulgaricus*, showed limited inhibition of *Enterococcus* sp. and *Listeria monocytogenes* strains, and was inactive against staphylococci. The activity spectrum of thermophilin 110, with the exception of pediococci, is

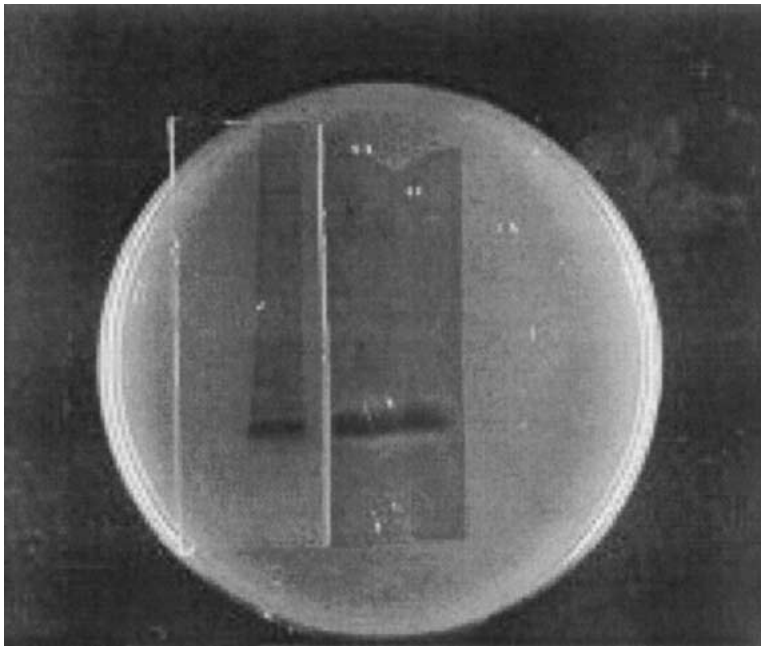

Fig. 5. Direct detection of thermophilin 110 by the agar overlay assay: (*left*), marker gel stained with Coomassie blue R-250; (*right*), polyacrylamide gel overlaid with MRS agar film seeded with *Pediococcus acidilactici* F.

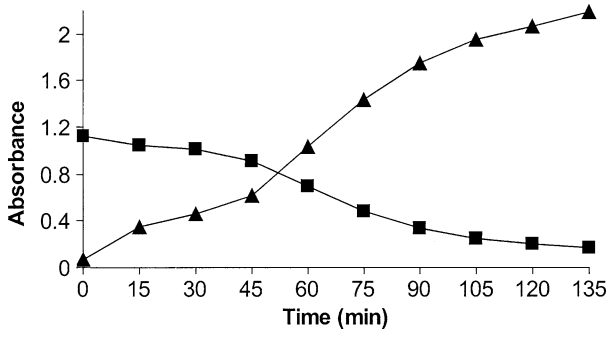

Fig. 6. Effect of inhibitory activity of thermophilin 110 on cell suspensions of *Pediococcus acidilactici* F. (■) Optical density at 660 nm. (▲) Optical density at 260 nm.

similar to other bacteriocins described in *S. thermophilus*, such as thermophilin 347 [37], thermophilin 13 [20], and the bacteriocin of *S. thermophilus* 81 [16], which included antilisterial activity. Because the presence of plasmid DNA in *S. thermophilus* ST110 could not be demonstrated, the genes involved in controlling bacteriocin production in this strain are assumed to be located on the chromosome.

The protein nature of thermophilin 110 was established by its sensitivity to several proteolytic enzymes. When thermophilin 110 was incubated with α-amylase, its antimicrobial activity was lost, which indicated the requirement of a glycosidic moiety for full activity. In this regard, thermophilin 110 was similar to thermophilin A [38], thermophilin T [2], and the bacteriocin of *S. thermophilus* 580 [25].

Thermophilin appears to be a low molecular weight two-component (based on SDS-PAGE) or possibly a three-component (based on MALDI-TOF-MS) peptide complex, estimated to contain approximately 16% carbohydrate by weight. The size of each of the two components detectable by gel electrophoresis is around 4–5 kDa, while the sizes of the three putative peptides detected by MALDI-TOF-MS analysis correspond to 5,139, 5,285, and 5,776 Da. Although two-peptide bacteriocins have been reported in all major groups of lactic acid bacteria [15], thermophilin 13 has been the only other known two-peptide bacteriocin produced by a strain of *S. thermophilus* [20].

The production of thermophilin 110 was related to the growth phase of the culture and highest bacteriocin levels were obtained in the late log or early stationary phase. The extension of incubation to 24 h resulted in a significant loss of bacteriocin activity that was similar to results reported by Ward and Somkuti for thermophilin A [38] and by Aktypis et al. for thermophilin T [2].

The mode of action of most bacteriocins produced by lactic acid bacteria involves primarily the permeabilization of cell membranes leading to the loss of cell viability [7]. However, selected strains of lactococci have been reported to produce bacteriocins that cause cell lysis in closely related target organisms [21, 22, 26]. In fact, the use of lactococci producing bacteriolytic bacteriocins as adjunct cultures in cheesemaking has been recommended to promote the release of intracellular enzymes by starter cultures and the acceleration of the ripening process [21, 29]. The exposure of susceptible strains of pediococci to

thermophilin 110 is characterized by cell lysis, which invites the evaluation of the potential of this bacteriocin in a variety of applications. Although strains of *P. acidilactici* and *P. pentosaceus* are used as starter cultures in food biotechnology (production of fermented sausage and vegetable products) and the bacteriocin of these cultures, pediocin, has been suggested for use as a natural biopreservative for *Listeria* control [32, 35], certain pediococci are also important spoilage microorganisms in wine [5] and beer [3, 12, 17] production. To control pediococci in the brewing industries, the efficacy of the GRAS (''generally recognized as safe'')-category lactococcal lantibiotic nisin has been investigated [11, 27, 28, 30]. It is possible that thermophilin 110, also a natural antimicrobial product of a food-grade fermentation bacterium, alone or in combination with the lantibiotic nisin produced by *L. lactis* subs. *lactis*, may also find applications in the brewing industries to control the growth of spoilage-causing pediococci. Furthermore, it may be possible to use the thermophilin 110-producing *S. thermophilus* strain as an adjunct culture to induce lysis of *P. acidilactici* strains used as starter cultures and to accelerate the curing of fermented meat and vegetable products.

In recent years, pediococci have also been detected in association with human diseases [1]. Although pediococci have not been identified as causative agents of any particular infectious disease, *P. acidilatici* and *P. pentosaceus* have been isolated in cases of bacteremia [10, 24, 31]. Because clinical isolates of pediococci may be naturally resistant to vancomycin [4, 14, 24], the possible involvement of pediococci as opportunistic pathogens in human infections provides an additional perspective for considering the evaluation of target-specific agents such as thermophilin 110 to control the growth of these microorganisms.

Studies are in progress to determine the primary structure and the contribution to antimicrobial activity of each component of the thermophilin 110 peptide complex. The possible activation of autolysin(s) in pediococci by thermophilin 110 that may be involved in cell lysis also requires further investigation.

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